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Molecular Structure and Enzymatic Function of Lycopene Cyclase from the Cyanobacterium *Synechococcus* sp Strain PCC7942

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A gene encoding the enzyme lycopene cyclase in the cyanobacterium *Synechococcus* sp strain PCC7942 was mapped by genetic complementation, cloned, and sequenced. This gene, which we have named *crtL*, was expressed in strains of *Escherichia coli* that were genetically engineered to accumulate the carotenoid precursors lycopene, neurosporene, and ζ-carotene. The *crtL* gene product converts the acyclic hydrocarbon lycopene into the bicyclic β-carotene, an essential component of the photosynthetic apparatus in oxygen-evolving organisms and a source of vitamin A in human and animal nutrition. The enzyme also converts neurosporene to the monocyclic β-zeacarotene but does not cyclize ζ-carotene, indicating that desaturation of the 7-8 or 7′-8′ carbon-carbon bond is required for cyclization. The bleaching herbicide 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA) effectively inhibits both cyclization reactions. A mutation that confers resistance to MPTA in *Synechococcus* sp PCC7942 was identified as a point mutation in the promoter region of *crtL*. The deduced amino acid sequence of lycopene cyclase specifies a polypeptide of 411 amino acids with a molecular weight of 46,125 and a pI of 6.0. An amino acid sequence motif indicative of FAD utilization is located at the N terminus of the polypeptide. DNA gel blot hybridization analysis indicated a single copy of *crtL* in *Synechococcus* sp PCC7942. Other than the FAD binding motif, the predicted amino acid sequence of the cyanobacterial lycopene cyclase bears little resemblance to the two known lycopene cyclase enzymes from nonphotosynthetic bacteria. Preliminary results from DNA gel blot hybridization experiments suggest that, like two earlier genes in the pathway, the *Synechococcus* gene encoding lycopene cyclase is homologous to plant and algal genes encoding this enzyme.

INTRODUCTION

The symmetrical, bicyclic carotenoid β-carotene is an essential component of the photosynthetic apparatus in oxygenic photosynthetic organisms (e.g., cyanobacteria, algae, and plants; Goodwin, 1980). This yellow pigment is intimately associated with the photosynthetic reaction centers and plays a vital role in protecting against potentially lethal photooxidative damage (Koyama, 1991). β-Carotene and other carotenoids derived from it also serve as light-harvesting pigments (Siefermann-Harms, 1987), are involved in the thermal dissipation of excess light energy captured by the light-harvesting antenna (Demmig-Adams and Adams, 1992), provide substrate for the biosynthesis of the plant growth regulator abscisic acid (Parry and Horgan, 1991; Rock and Zeevaart, 1991), and are precursors of vitamin A in human and animal diets (Krinsky, 1987). Plants also exploit carotenoids as coloring agents in flowers and fruits to attract pollinators and agents of seed dispersal (Goodwin, 1980), and the color provided by carotenoids is of agronomic value in a number of important crops. Despite many attempts, the enzyme or enzymes catalyzing the formation of the bicyclic β-carotene from the acyclic precursor lycopene have not been isolated from any photosynthetic organism, nor have the corresponding genes been identified and sequenced or the cofactor requirements established.

As illustrated in Figure 1, the production of the symmetrical 40 carbon phytoene from geranylgeranyl pyrophosphate is the first step specific to the pathway of carotenoid biosynthesis (Spurgeon and Porter, 1980). Phytoene then undergoes a series of four desaturation steps to form first phytofluene and then, in turn, ζ-carotene, neurosporene, and lycopene. Cyclization reactions at each end of the lycopene molecule result in the formation of β-carotene, which may then serve as the substrate for production of the xanthophylls or oxygenated carotenoids that are also important constituents of the

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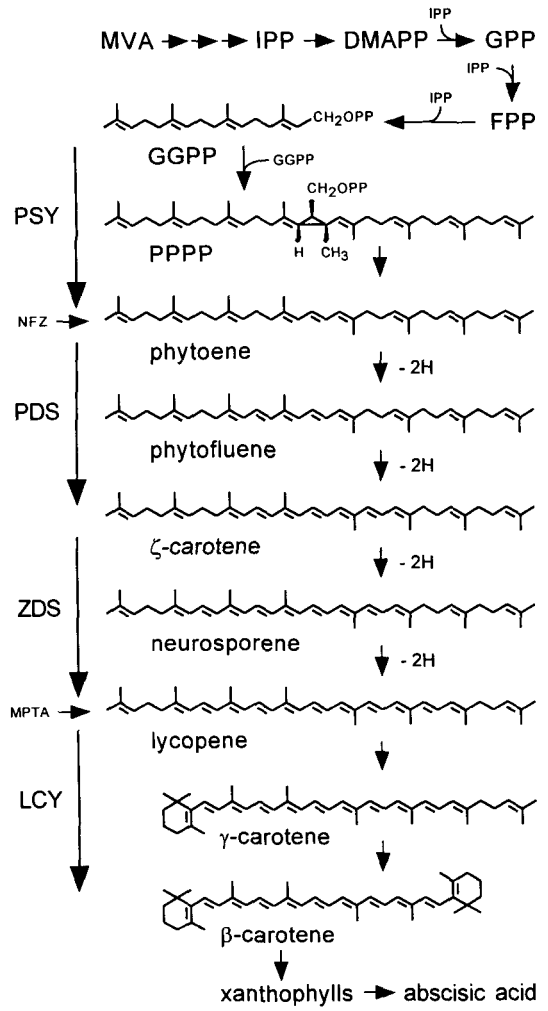

Figure 1. Pathway of Carotenoid Biosynthesis in Cyanobacteria and Plants.

The enzymes catalyzing various steps are indicated at the left. Target sites of the bleaching herbicides norflurazon (NFZ) and 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA) are also indicated at the left. DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; LCY, lycopene cyclase; MVA, mevalonic acid; PDS, phytoene desaturase; PPPP, prephytoene pyrophosphate; PSY, phytoene synthase; ZDS, ζ-carotene desaturase.

photosynthetic membrane. All of the enzymes catalyzing these reactions, as well as the carotenoids themselves, are thought to be membrane bound (see Bramley, 1985).

The transformable cyanobacterium *Synechococcus* sp strain PCC7942 has proven to be a useful model organism for the study of the carotenoid biosynthetic pathway in oxygenic photosynthetic organisms. The identification and cloning of the gene encoding phytoene desaturase (*crtP*, formerly called *pds*) in *Synechococcus* (Chamovitz et al., 1991) enabled the cloning and characterization of heretofore unidentified and homologous phytoene desaturase genes from algae and plants (Bartley et al., 1991; Pecker et al., 1992, 1993). Following the strategy that had proven successful for the phytoene desaturase gene, we recently located a *Synechococcus* gene encoding an enzyme that converts lycopene to β-carotene (Cunningham et al., 1993). Mutants of *Synechococcus* were selected for resistance to an experimental herbicide, 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA), that inhibits the synthesis of carotenoids with cyclic end groups and is thought to act by interfering with the function of the lycopene cyclase enzyme (see Sandmann and Böger, 1989). One such mutation was mapped by genetic complementation, and subsequent experiments demonstrated that the genetic information for a lycopene cyclase enzyme resides in the vicinity of this mutation (Cunningham et al., 1993). We have now analyzed the DNA sequence in this region and identified both the gene encoding lycopene cyclase (*crtL*, formerly called *lcy*) and a mutation responsible for resistance to MPTA. Here, we present the molecular analysis of *crtL* and its polypeptide product and then examine the substrate specificity of the enzyme by expressing the gene in cells of *Escherichia coli* engineered to accumulate lycopene, neurosporene, or ζ-carotene.

RESULTS

Phenotypic Complementation Mapping of Lycopene Cyclase Activity

In an earlier publication (Cunningham et al., 1993), we described the genetic mapping of a mutation, MPTA^r-5, that confers resistance to the bleaching herbicide MPTA in the cyanobacterium *Synechococcus* sp PCC7942. We also demonstrated that the gene *crtL* (formerly *lcy*), encoding the enzyme lycopene cyclase, resides near this mutation. Additional mapping experiments, illustrated in Figure 2, define the orientation of *crtL* and indicate that the 5′ end of the coding region is in the immediate vicinity of the mutation. These experiments were performed by first cloning genomic fragments of *Synechococcus* sp PCC7942 in the vector pTrcHisB and then introducing the resulting plasmids into a strain of *E. coli* that accumulates lycopene and consequently forms pink colonies (see Methods). Because β-carotene is yellow, the appearance of pinkish-yellow or yellow colonies after transformation provides a simple visual demonstration of lycopene cyclase activity. HPLC analysis of pigment extracts was used to confirm these observations.

The smallest genomic DNA fragment producing lycopene cyclase activity in *E. coli* was a 1.5-kb PstI-PstI fragment that also contained the MPTA^r-5 mutation. However, the activity provided by this clone was low, and only approximately half of the lycopene in *E. coli* was converted to β-carotene. A slightly larger KpnI-SalI clone provided the maximum activity. The cloning frame was not important because lycopene cyclase activity

was also observed for the KpnI-SalI and PstI-PstI fragments cloned in the other two frames (using pTrcHisA and pTrcHisC). The data shown in Figure 2 are for genomic clones of the wild-type strain of *Synechococcus* sp PCC7942, but clones from MPTA-resistant mutant MPTA^r-5 provided comparable lycopene cyclase activity.

The dependence of enzymatic activity on the orientation of the genomic fragments (Figure 2) but not on the cloning frame indicates that the cyanobacterial promoter of *crtL* is not well utilized by the *E. coli* transcriptional machinery. Rather, the pTrcHis promoter and/or other upstream elements of the vector are, in some way, required for the production of the *Synechococcus* lycopene cyclase in *E. coli.* The choice of pTrcHisB as vector was, in fact, fortuitous because the same *Synechococcus* genomic DNA fragments cloned in the vectors pBluescript II KS+ and SK+ provided little enzymatic activity regardless of orientation, cloning frame, or inclusion of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) (data not shown).

Identification of Mutation MPTA^r-5, *crtL*, and Nearby Open Reading Frames

DNA sequence analysis revealed four potential open reading frames (ORFs) in a 4.6-kb EcoRI-BamHI genomic DNA fragment encompassing the MPTA^r-5 mutation and the lycopene cyclase activity in *Synechococcus* sp PCC7942. An analysis

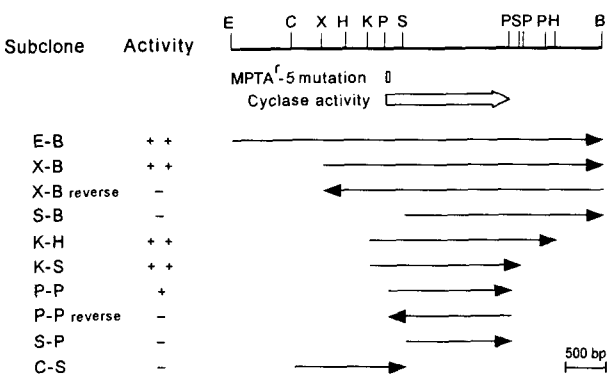

Figure 2. Complementation Mapping of Lycopene Cyclase Activity.

Also shown is the previously mapped location of a mutation, MPTA^r-5, that confers resistance to the bleaching herbicide MPTA. Arrows indicate the genomic DNA fragments of *Synechococcus* sp PCC7942 that were inserted in the expression vector pTrcHisB and their orientation relative to the promoter in the plasmid. Lycopene cyclase activity was ascertained by observation of colony color after the indicated subclones were introduced into cells of *E. coli* that were engineered to accumulate lycopene. Cyclase activity was scored as (−) for pink colonies indistinguishable from controls containing the empty vector pTrcHisB, (+) for yellow colonies retaining a strong pink hue, and (++) for deep yellow colonies with no hint of pink. HPLC analysis was used to confirm the visual observations. E, EcoRI; C, ClaI; X, XbaI; H, HindIII; K, KpnI; P, PstI; S, SalI; B, BamHI.

of codon usage compared to that of other *Synechococcus* genes (data not shown) indicated that all four ORFs are good candidates for genes that are transcribed in *Synechococcus.* The four ORFs are displayed schematically in Figure 3.

ORF 3 is entirely coincident with the location mapped for the lycopene cyclase activity and is in the orientation expected. DNA gel blot hybridization analysis of *Synechococcus* genomic DNA under conditions of low stringency indicated that there is only one copy of the ORF 3 gene in this organism (data not shown). We have named this gene *crtL* in accordance with recently proposed rules of nomenclature for carotenoid biosynthetic genes (Hirschberg and Chamovitz, 1994).

The genetic lesion in herbicide-resistant mutant MPTA^r-5 is a point mutation that lies 106 bp upstream of the predicted initiation codon of *crtL.* The mutation is within the ~50-bp region previously defined by complementation mapping of MPTA resistance (Cunningham et al., 1993; see Figures 2 and 3). No other mutations were found in the course of sequencing the entire ORF 3 of mutant MPTA^r-5 and as far upstream as the XbaI site preceding ORF 2. Though it occurs in the coding region of ORF 2, the mutation does not alter the predicted amino acid sequence of the protein encoded by this ORF (Figure 3).

The function and identity of ORF 2 remain unknown. A search of amino acid and nucleic acid sequence data bases uncovered no protein or DNA sequences with appreciable similarity to this putative gene or its predicted 258 amino acid (28,273 molecular weight) polypeptide, nor were any motifs or other notable features discerned. Because genes for a given pathway are often clustered in cyanobacteria, the position of ORF 2 immediately upstream of and in the same orientation as *crtL* is suggestive of a role in carotenoid biosynthesis. However, interruption of the ORF 2 sequence by insertion of the aminoglycoside 3′-phosphotransferase gene of Tn*903* in the HindIII site did not measurably affect either viability or pigmentation of *Synechococcus* (F.X. Cunningham, Jr. and E. Gantt, unpublished data).

The predicted product of ORF 1 is 333 amino acids in length with a molecular weight of 37,001. Its sequence is similar (~52% identity with three gaps) to the small subunit of phenylalanine tRNA synthetase encoded by the *pheS* gene of *E. coli* (Fayat et al., 1983; Swiss-Prot accession number P08312), with slightly less sequence identity to gene products of *Bacillus subtilis* (Brakhage et al., 1990; P17921) and *Thermus thermophilus* (Keller et al., 1992; Kreutzer et al., 1992; P27001). The deduced amino acid sequence of the *Synechococcus* gene also contains the two motifs expected for a phenylalanine tRNA synthetase (Eriani et al., 1990).

The putative polypeptide product of ORF 4 is 222 amino acids in length with a molecular weight of 24,313. It is nearly 48% identical (with three gaps) to a hypothetical 230 amino acid polypeptide predicted by an ORF in the nonphotosynthetic bacterium *Pseudomonas aeruginosa* (ORF 5 of Whitchurch et al., 1991; Swiss-Prot P24562). Insertional inactivation of the *P. aeruginosa* gene was reported to have no discernable effect on growth (Savioz et al., 1993).

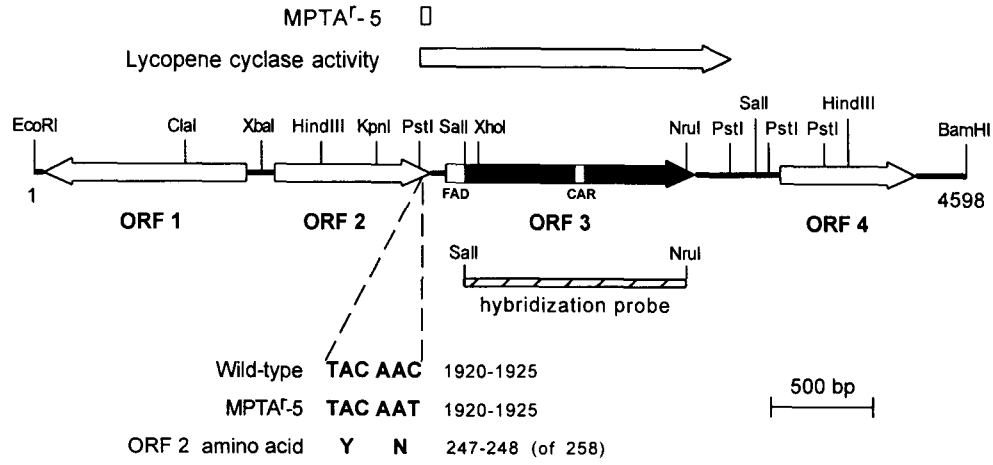

Figure 3. Physical Map of and ORFs in a 4598-bp EcoRI-BamHI Genomic DNA Fragment of *Synechococcus* sp PCC7942.

Mapped locations of a gene encoding lycopene cyclase activity and a mutation (MPTA^r-5) endowing resistance to the bleaching herbicide MPTA are indicated above the map. A SalI-NruI probe used for hybridization analysis of ORF 3 is indicated below it. A point mutation that defines the mutant MPTA^r-5 is displayed. Two regions of interest in the polypeptide encoded by ORF 3 are indicated by open boxes labeled FAD and CAR.

Molecular Structure of the *crtL* Gene Product

The DNA sequence and predicted amino acid sequence of *crtL* (ORF 3) are presented in Figure 4. The coding region commences with the much less frequently used GTG at bases 181 to 183. The similarity of the codon usage in this region to that in other known genes of *Synechococcus* (data not shown), the position of the GTG relative to a prospective ribosome binding site (Figure 4), an FAD binding motif at the N terminus of the predicted polypeptide (Figure 5A), and the lack of an ATG in-frame in this vicinity together support the choice of this GTG as the initiation codon for *crtL*.

The deduced amino acid sequence of *crtL* predicts a polypeptide product of 411 amino acid residues with a molecular weight of 46,125 and a pI of 6.0. No genes or polypeptides with significant resemblance to the *crtL* gene or gene product were discovered in searches of DNA and protein sequence data bases. Two local features of interest in the predicted amino acid sequence are underlined in Figure 4 and are compared to similar sequences of other enzymes in Figure 5.

The primary structure of the N-terminal end of the *crtL* gene product conforms to an amino acid sequence motif defined for polypeptides that bind such dinucleotides as NADP, NAD, and FAD. This region of the molecule is believed to fold into a characteristic β sheet–α helix-loop-β sheet configuration referred to as the Rossmann or dinucleotide binding fold (Rossmann et al., 1974; Wierenga et al., 1986). An earlier specification of this motif involved 11 different amino acid positions (Wierenga et al., 1986). A more detailed and slightly modified motif, specifically for FAD binding proteins and based on 20 examples (Van Beeumen et al., 1991), defines six more positions. This FAD binding signature is listed in Figure 5A above the predicted amino acid sequences of lycopene cyclase and phytoene desaturase (*crtP* gene product) from *Synechococcus*, and those of the other two known lycopene cyclase genes (*crtY* genes) from the nonphotosynthetic bacteria *Erwinia herbicola* and *E. uredovora*.

None of the four sequences displayed in Figure 5A fits all 17 specifications of the FAD motif, but all closely approximate it, each conforming to 15 of the 17 criteria. The differences are mostly conservative, and this motif must be considered a flexible and evolving one because a number of exceptions have been noted since it was defined by Van Beeumen et al. (1991). For instance, the serine of the *Synechococcus* sp PCC7942 lycopene cyclase at position 7 of the motif, normally an alanine or glycine, can be considered a relatively conservative substitution and has been reported for the FAD binding sequences of several other enzymes (McKie and Douglas, 1991; Claiborne et al., 1992). The glycine of *Synechococcus* lycopene cyclase at position 29 also does not fit the motif, but other exceptions have been reported for this position in FAD-utilizing enzymes (see Wierenga et al., 1986; Van Beeumen et al., 1991).

The second feature of interest observed in the amino acid sequence of *Synechococcus* lycopene cyclase is at positions 212 to 229. This region conforms to the definition of a motif defined for substrate carrier proteins involved in energy transfer in the inner mitochondrial membrane (see Klingenberg, 1990; Walker, 1992). We also observed that this motif is present, or nearly so, in an earlier enzyme of the carotenoid biosynthetic pathway of oxygenic photosynthetic organisms, namely phytoene desaturase (Figure 5B). The similarity between phytoene desaturase and lycopene cyclase in this region is more extensive than that specified by the mitochondrial carrier motif. The mitochondrial carrier motif is not present in deduced amino acid sequences of the published bacterial phytoene desaturase and lycopene cyclase genes.

The appearance of a mitochondrial-type sequence in

lycopene cyclase and phytoene desaturase enzymes does not necessarily imply a relationship to the mitochondrial carrier proteins. The signature for the mitochondrial proteins actually consists of three tandem repeats of a domain of ~100 residues that contains the specified motif. The functional implications of the limited sequence similarity between lycopene cyclase and phytoene desaturase, if indeed there are any, are unknown. What these two enzymes have in common are that both are membrane-associated polypeptides, each probably utilizes FAD as a cofactor, and their respective substrates, lycopene and phytoene (Figure 1), are structurally similar.

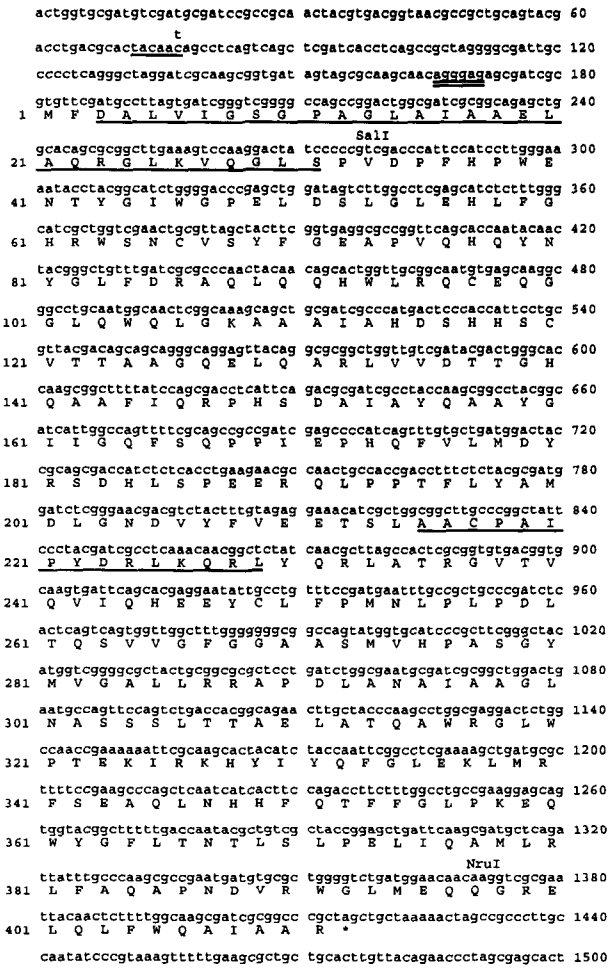

Figure 4. DNA Sequence and Predicted Amino Acid Sequence of the *crtL* Gene of *Synechococcus* sp PCC7942.

DNA bases are numbered at the right and amino acids at the left. The point mutation of mutant MPTA^r-5 is indicated above the wild-type DNA sequence at position 77, and a putative −10 or Pribnow box that contains this base is underlined (bases 72 to 77). A prospective ribosome binding site is double-underlined (bases 166 to 171). An FAD binding motif in the amino acid sequence (amino acids 3 to 31) and a region with similarity to phytoene desaturase genes of algae, plants, and cyanobacteria (amino acids 215 to 229) are underlined. The SalI and NruI sites indicated above the sequence define the limits of a DNA fragment used as a hybridization probe.

Carotenoid biosynthesis from phytoene to β-carotene (Figure 1), in keeping with the nonpolar nature of the hydrocarbon substrates, is a pathway localized in membranes (Bramley, 1985). Yet, surprisingly, the predicted *Synechococcus* sp PCC7942 *crtL* gene product is not particularly hydrophobic, with an average hydrophobic index of −0.18 and with 25% of the amino acids being charged ones. The *Synechococcus* phytoene desaturase is even more highly charged (29% of the amino acids). A plot of the hydropathic index of the amino acid sequence of *Synechococcus* lycopene cyclase is displayed in Figure 6. There are several hydrophobic regions with apparent potential to span the membrane, but none approaches a hydrophobicity that would indicate a high probability of it (e.g., a value of greater than +1.6 averaged over a window of 19 residues; Kyte and Doolittle, 1982; note that a window of 11 amino acids was used for Figure 6 to provide more fine structural detail).

Substrate Specificity of the Cyclization Reaction

A KpnI-HindIII fragment containing the *Synechococcus* sp PCC7942 *crtL* gene was cloned in pTrcHisA to give the plasmid pTrcA-LCYKH illustrated in Figure 7. This plasmid engenders the biosynthesis of a functional lycopene cyclase enzyme in cells of *E. coli* that contain it (e.g., see Figure 2). To examine the substrate specificity of the lycopene cyclase enzyme, we constructed the plasmids pAC-ZETA, pAC-NEUR, and pAC-LYC (see Methods for details), such that cells of *E. coli* containing them accumulate the carotenoid pigments ζ-carotene, neurosporene, and lycopene, respectively. The plasmid pTrcA-LCYKH was introduced into these ζ-carotene-, neurosporene-, and lycopene-accumulating strains of *E. coli*, with the results shown in Figure 8.

Figure 8A illustrates HPLC elution profiles of pigment extracts from "control" cultures that contained the empty cloning vector pTrcHisA in addition to the indicated pAC plasmid. The HPLC elution profiles are indistinguishable from those of cultures lacking pTrcHisA (data not shown) and display predominantly a single band, which has the retention time and absorption spectrum expected for lycopene (peak 1), neurosporene (peak 2), or ζ-carotene (peak 3). When plasmid pTrcA-LCYKH, expressing the *Synechococcus crtL* gene product, was introduced in place of pTrcHisA, the lycopene and neurosporene peaks were reduced or eliminated and new elution peaks appeared (Figure 8B, left and center profiles). In contrast, the elution profile of the culture accumulating ζ-carotene was not affected by the introduction of plasmid pTrcA-LCYKH (Figure 8B, profile at right), and the spectrum of the major peak (peak 3′; spectrum not shown) is indistinguishable from that of ζ-carotene (peak 3; spectrum at right in Figure 8D). We would not expect cyclization of ζ-carotene to materially affect the absorption spectrum because the planarity of the conjugated system of double

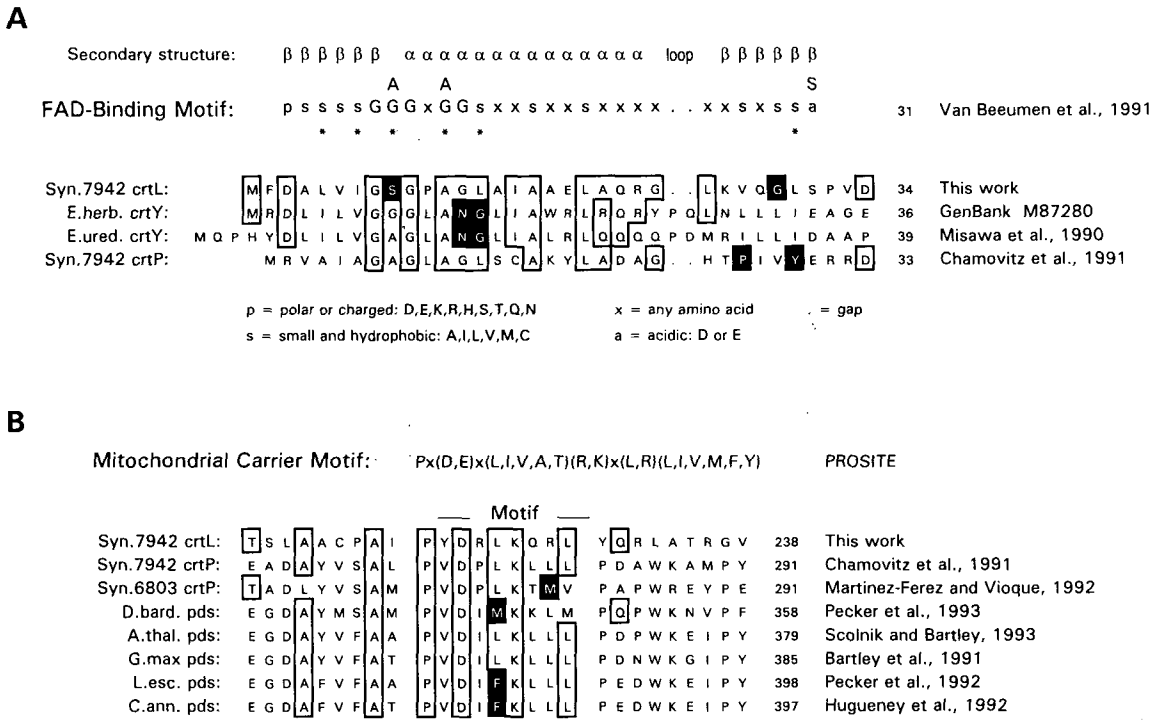

Figure 5. Comparison of a Putative FAD Binding Motif and a Possible Substrate Carrier Motif in the *Synechococcus* sp PCC7942 Lycopene Cyclase to Similar Sequences in Other Enzymes of Carotenoid Biosynthesis.

Residues identical in the *Synechococcus* lycopene cyclase and one or more of the other enzymes are boxed. Amino acid residues that do not conform to the motifs are in white type on a black background.

(A) The N terminus of the *Synechococcus* sp PCC7942 lycopene cyclase gene product (Syn.7942 crtL) is compared with those in products of the two other known lycopene cyclase genes from *E. herbicola* (E.her. crtY) and *E. uredovora* (E.ured. crtY, GenBank accession number D90087), and that in the product of the *Synechococcus* sp PCC7942 phytoene desaturase gene (Syn.7942 crtP, accession number X55289). A motif conserved in enzymes that bind FAD is defined above the sequences. Asterisks are placed beneath the six positions that differentiate the FAD binding motif from a more general dinucleotide one. The motif includes a variable loop region that may range from two to four amino acids.

(B) A region of the *Synechococcus* sp PCC7942 lycopene cyclase that conforms to a motif conserved in mitochondrial substrate carrier proteins is compared to similar regions in phytoene desaturase gene products of cyanobacteria (*crtP* genes) and algae and plants (*pds* genes). Abbreviations and GenBank accession numbers are as follows: Syn.6803, *Synechocystis* sp strain PCC6803, X62574; D.bard., *Dunaliella bardawil* (no GenBank accession number); G.max, *Glycine max*, M64704; A.thal., *Arabidopsis thaliana* (strain Columbia), L16237; L.esc., *Lycopersicon esculentum*, X59948; C.ann., *Capsicum annuum*, X68058.

bonds that constitute the chromophore would not be significantly affected. However, we do expect that cyclization would produce a significant and easily detected change in the HPLC retention time as is observed after cyclization of neurosporene and lycopene.

The new band (peak 4) appearing in the HPLC elution profile of the pigment extract from the culture containing pTrcA-LCYKH plus pAC-LYC has the retention time of authentic β-carotene and has the absorption spectrum (Figure 8D, profile at left) expected for this carotenoid pigment. A group of at least five peaks (peaks 5 to 9; Figure 8B, middle profile) appears in place of neurosporene in cultures containing pAC-NEUR plus pTrcA-LCYKH. However, all of these new elution peaks exhibit absorption spectra similar to that expected for β-zeacarotene (Figure 9; see Davies, 1976) and are probably *cis* geometrical isomers that result from rotations about the many double bonds of this compound. Other HPLC peaks (e.g., peaks 1 to 4) are also likely to be comprised of mixtures of geometric isomers (e.g., see Linden et al., 1991). The resolution and complexity of the β-zeacarotene isomer mixture probably result from the asymmetrical nature of this compound. For the symmetrical β-carotene, ζ-carotene, or lycopene, a *cis* isomer resulting from a rotation about the 9-10 carbon-carbon double bond is identical to one obtained by rotation about the 9′-10′ carbon-carbon double bond (see Figure 10 for numbering of the carbon atoms). For the asymmetrical β-zeacarotene, two distinctly different isomers would result and, depending on how close the *cis* double bond is to the ring, very different effects on retention time would be expected.

The bleaching herbicide MPTA effectively inhibits both cyclization of lycopene and of neurosporene at a concentration of 40 μM (Figure 8C, left and middle profiles). A trace of

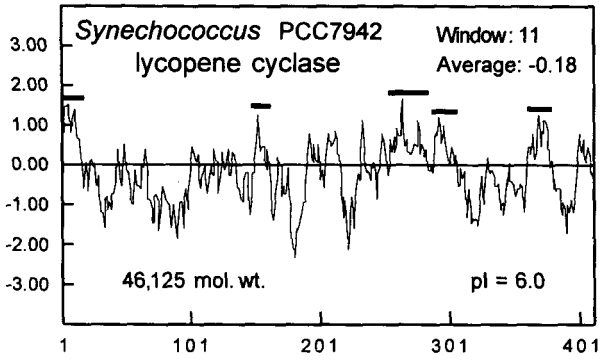

Figure 6. Hydrophobicity Plot for the *Synechococcus* sp PCC7942 Lycopene Cyclase.

Potential transmembrane regions are marked with a bold horizontal line above the plot. The plot was created using values for amino acid hydropathy reported by Kyte and Doolittle (1982).

β-carotene and the monocyclic intermediate, γ-carotene (with retention time intermediate to lycopene and β-carotene) remain in the pAC-LYC plus pTrcA-LCYKH cultures (Figure 8C, profile at left), indicating that inhibition is not quite complete (see also Cunningham et al., 1993). The accumulation of ζ-carotene in cells containing pAC-ZETA was not affected by MPTA at this (Figure 8C, profile at right) or at much higher concentrations (100 and 250 μM; data not shown).

DISCUSSION

Molecular Basis of Resistance to MPTA in Mutant MPTA^r-5

Our results provide a direct demonstration that the enzyme lycopene cyclase is the target site of the bleaching herbicide MPTA. A molecular lesion conferring resistance to MPTA was identified as a point mutation 106 bp upstream of the start codon of the *Synechococcus crtL* gene (Figures 3 and 4). This single alteration in sequence allows cultures of *Synechococcus* to tolerate the presence of more than 100 μM MPTA, whereas the wild type will not grow when the concentration is 2 μM or above (Cunningham et al., 1993).

The replacement of a C residue in the wild type with a T residue in mutant MPTA^r-5 produces a six-base sequence (TACAAT from TACAAC) that is closer to the −10 or Pribnow box consensus sequence in the promoter of *E. coli* genes (TATAAT; Hawley and McClure, 1983). Mutations in *E. coli* which bring the Pribnow box closer to the consensus sequence typically result in enhanced transcription (Hawley and McClure, 1983). The T residue in position six of the consensus *E. coli* promoter is the most highly conserved residue (96% in 112 genes examined) and is presumed to be a major determinant of promoter strength. The remaining difference in the *Synechococcus* sequence, the C in position three, is in the least highly conserved residue (44%) of the *E. coli* consensus sequence.

Given these considerations, we tentatively conclude that resistance to MPTA in Synechococcus mutant MPTA^r-5 derives from the overexpression of the *crtL* gene. A similar phenomenon has been described for a fluridone-resistant mutant of *Synechococcus* sp PCC7942 (mutant FD5) where a deletion mutation altered the promoter of the phytoene desaturase gene and resulted in at least a 20-fold increase in the level of the phytoene desaturase enzyme as measured by immunoblotting (Chamovitz et al., 1993). We predict that mutant MPTA^r-5 will contain a greatly elevated level of the cyclase enzyme and that it will also be tolerant of other herbicides that inhibit lycopene cyclase activity. This prediction will be tested when specific antibodies become available.

Substrate for the Cyclization Reaction

There are two different routes by which β-carotene could be synthesized from neurosporene. These are illustrated in Figure

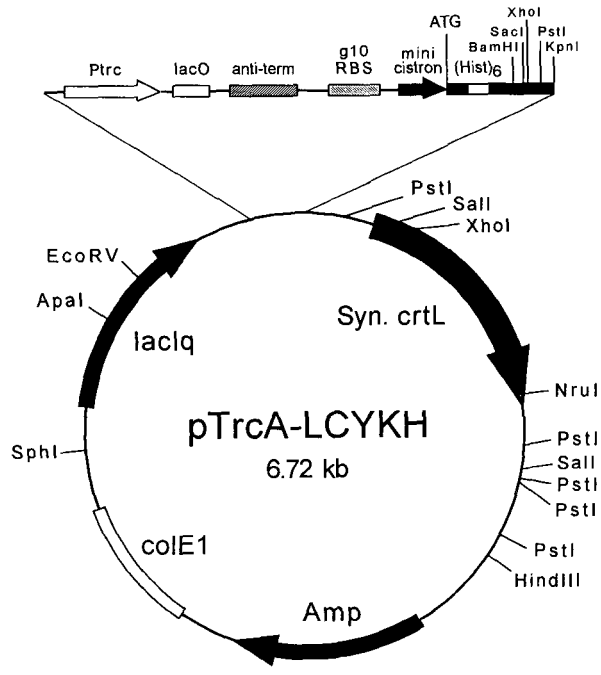

Figure 7. Structure of Plasmid pTrcA-LCYKH with Upstream Elements of the Cloning Vector Displayed in Magnification.

The *Synechococcus* (Syn.) *crtL* gene is not fused to the IPTG-inducible N-terminal peptide immediately downstream of the minicistron, but the upstream elements of the vector greatly enhance production of the enzymatically active *crtL* gene product. Amp, ampicillin resistance gene; anti-term, *E. coli rrnB* antiterminator; RBS, ribosome binding site from bacteriophage T7 gene 10.

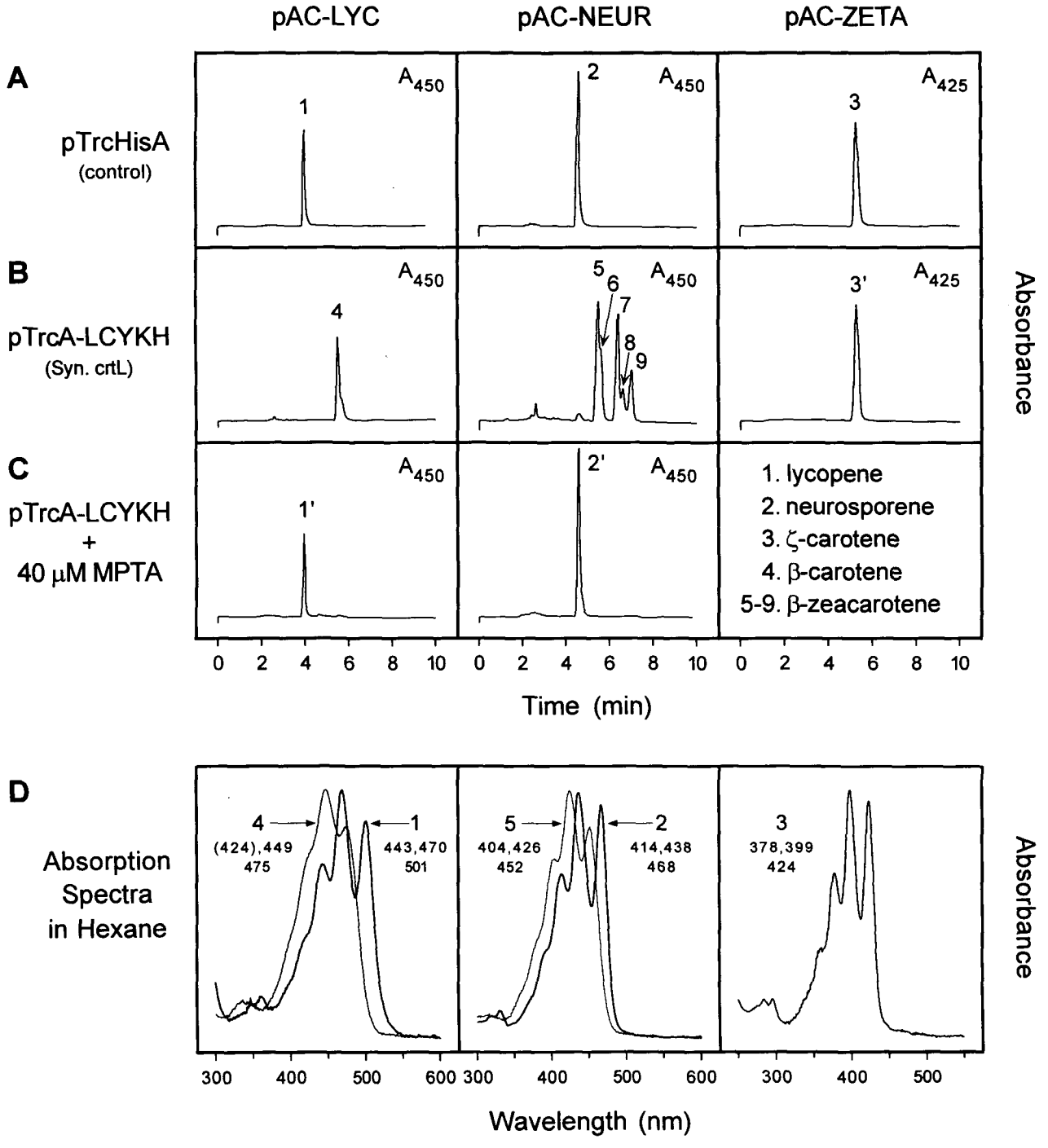

Figure 8. HPLC Elution Profiles of Pigments Extracted from Cultures of *E. coli* Containing the Plasmids Indicated (Above and to the Left) and Treated with the Bleaching Herbicide MPTA Where Indicated.

Detection was at 450 or 425 nm for **(A)**, **(B)**, and **(C)** as indicated.

(A) Elution profiles of control cultures containing the empty vector pTrcHisA in addition to the plasmids pAC-LYC, pAC-NEUR, or pAC-ZETA.

(B) Elution profiles of cultures containing pTrcA-LCYKH and expressing the *Synechococcus crtL* gene (Syn. crtL) encoding lycopene cyclase. Cultures also contained either pAC-LYC, pAC-NEUR, or pAC-ZETA.

(C) As in **(B)** but with the addition of 40 μM MPTA to the culture media.

(D) Absorption spectra of numbered peaks in the elution profiles of **(A)**, **(B)**, and **(C)** are displayed along with their absorption maxima in hexane.

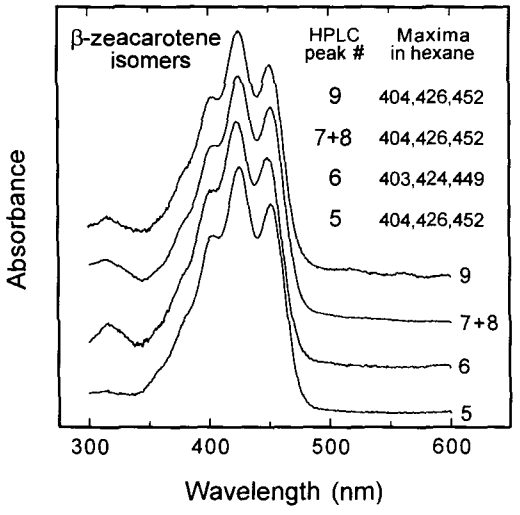

Figure 9. Absorption Spectra in Hexane of β-Zeacarotene Stereoisomers Produced after Expression of the *Synechococcus* sp PCC7942 *crtL* Gene in a Neurosporene-Accumulating Strain of *E. coli.*

HPLC peak numbers (5, 6, 7, 8, and 9) correspond to peak numbers in the HPLC elution profile displayed in the middle section of Figure 8B.

10. Which of these two routes is actually followed in vivo has been a matter of some discussion. Experimental data have been regarded as supporting the role of lycopene as the biological precursor of β-carotene (see Spurgeon and Porter, 1980; Jones and Porter, 1986). The evidence consists of observations that lycopene accumulates in the presence of cyclization inhibitors, such as nicotine and MPTA, and that the accumulated lycopene can be converted to β-carotene after the inhibitor is removed. Also, there are reports of cell-free preparations that can convert lycopene into β-carotene (reviewed in Spurgeon and Porter, 1980; see also Goodwin, 1980; Bramley, 1985).

The occurrence of β-zeacarotene in many plants and algae indicates that cyclization of neurosporene also can occur. In a most informative experiment, Bramley et al. (1977) observed that β-zeacarotene and lycopene added to cell-free extracts of the fungus *Phycomyces blakesleeanus* were equally effective in competing with isotopically labeled neurosporene for incorporation into β-carotene. These authors concluded that the two alternative routes are of equal importance in the fungal pathway.

In this report, we have demonstrated that the product of a single cyanobacterial gene, *crtL*, is sufficient to catalyze the two cyclizations required to make β-carotene from lycopene in a lycopene-accumulating strain of *E. coli.* Our data establish that the same lycopene cyclase enzyme also converts the incompletely desaturated neurosporene into the monocyclic β-zeacarotene in *E. coli* (Figures 8 and 9). It is important to note that the lycopene cyclase in this heterologous system is a product of the authentic *Synechococcus* gene and not a fusion protein that might have altered function and specificity as a result. Because ζ-carotene was not a substrate for the enzyme, we conclude, as was suggested by others (see Goodwin, 1980; Britton, 1988), that desaturation of a double bond at the 7,8 or 7',8' position (see Figure 10) is a prerequisite for cyclization.

The question of the preferred biological substrate, neurosporene or lycopene, may actually have little meaning. There is

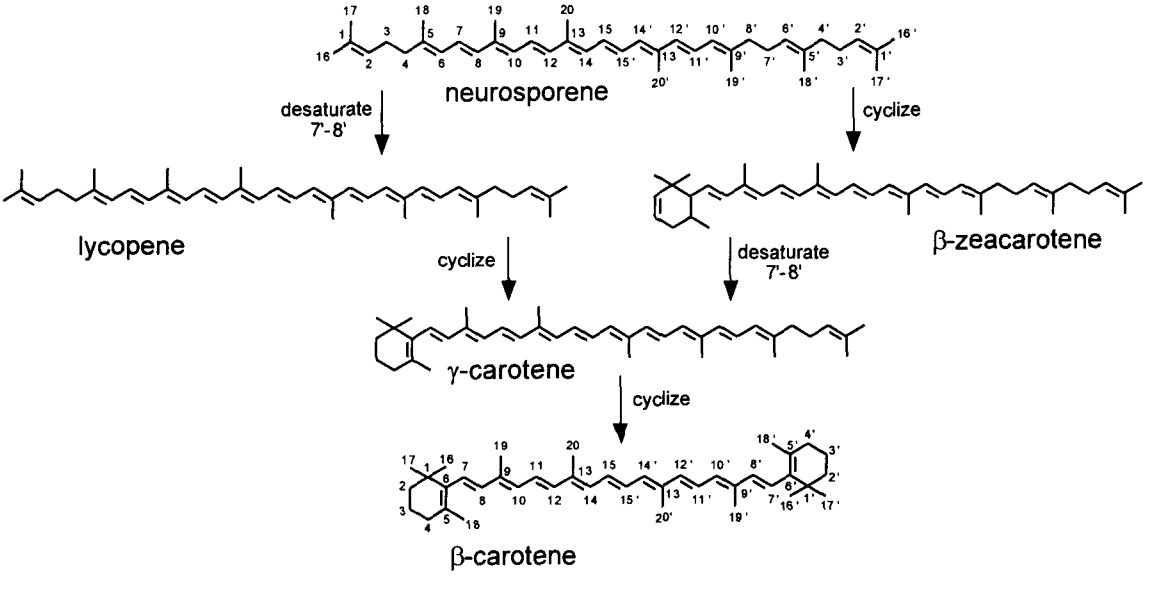

Figure 10. Alternative Routes of β-Carotene Biosynthesis from Neurosporene.

Conventional numbering of the carbon atoms (after Davies, 1976) is indicated for neurosporene and β-carotene.

experimental evidence for *P. blakesleeanus* (Candau et al., 1991) and numerous indications from studies of plants and other organisms (see Bramley, 1985) that multiple copies of the desaturases and lycopene cyclase are associated in a multienzyme complex. The "channeling" facilitated by such a complex could help to maximize production of the biologically functional β-carotene. Each half of the symmetrical phytoene molecule might then be desaturated and cyclized without reference to the other half of the molecule. In effect, the desaturation state of the half-molecule would be the relevant consideration.

Reaction Mechanism and Cofactor Requirements of Lycopene Cyclase

Relatively little is known about the enzymatic conversion of lycopene to β-carotene in plants or bacteria. Many attempts have been made to isolate enzymes that catalyze this reaction, and the work of Bramley and Taylor (1985), Camara and Dogbo (1986), and Beyer (1987) have demonstrated that detergent solubilization of these membrane-bound enzymes can be achieved. However, the activity is not particularly stable, and a purification to homogeneity has remained elusive. Studies done with crudely purified extracts have been inconclusive in determining what, if any, are the cofactor requirements. Cyclization of lycopene in detergent extracts or acetone powders of *Capsicum annuum* chromoplasts (Camara and Dogbo, 1986) was found not to require $NADP^+$ or FAD, whereas tomato cell-free extracts absolutely required FAD and were stimulated by $NADP^+$ (Kushwaha et al., 1969). A recent study examining the lycopene cyclase gene of *E. herbicola* (*crtY*) overexpressed in *E. coli* reported that only the all-*trans* isomer of lycopene was a substrate in crude cell-free lysates (Hundle et al., 1993). Cofactor requirements of this bacterial enzyme were not reported.

The proposed reaction mechanism for ring formation by lycopene cyclase involves a proton attack at carbon 2 of the acyclic precursor (see Figure 10 for numbering of the carbon atoms), followed by ring closure to yield a carbonium ion intermediate. This is then stabilized by loss of a proton to form the β-ring (Britton, 1988). Lycopene and β-carotene (and the monocyclic intermediate γ-carotene) have the same molecular formula ($C_{40}H_{56}$), as do the pair neurosporene and β-zeacarotene ($C_{40}H_{57}$). In effect, cyclization involves a rearrangement of hydrogen atoms mediated by a reduction at one portion of the molecule that is balanced by a concomitant oxidation at another location. The requirement for a double bond at the 7,8 (or 7′,8′) position indicates that this bond participates in the reaction, perhaps by stabilizing the carbonium intermediate. The putative FAD binding motif recognized in the primary structure of the *Synechococcus crtL* gene product suggests that lycopene cyclase is a flavoenzyme, and that FAD bound to the enzyme participates, in some way, in the cyclization reaction.

Comparison of Lycopene Cyclases from Photosynthetic and Nonphotosynthetic Organisms

The lycopene cyclase gene of *Synechococcus* is the only one yet identified and sequenced from a photosynthetic organism, but two bacterial genes encoding this enzyme have also been described. Like the *Synechococcus* gene product, those from the phytopathogenic bacteria *E. herbicola* (GenBank M87280) and *E. uredovora* (Misawa et al., 1990; see also GenBank M90698) also exhibit putative FAD binding motifs at the N terminus (see Figure 5), and the predicted sizes of the two bacterial enzymes (386 and 382 amino acids, respectively) are comparable to that predicted for the *Synechococcus* lycopene cyclase (411 amino acids). Also, like the *Synechococcus* enzyme, the two *Erwinia* enzymes are not particularly hydrophobic (26% of the amino acid residues charged versus 25% for the *Synechococcus* enzyme); however, their isoelectric points are greater than 9 while the pI for the *Synechococcus* enzyme is only 6.0. The deduced amino acid sequences of the two *Erwinia* gene products are 58% identical and 74% similar. A comparison of *Synechococcus* lycopene cyclase to the two *Erwinia* enzymes using the same settings for gap weight (3.0) and length weight (0.1) yields sequence identities of only 22 to 23% and similarities of 47% with 12 to 14 gaps. More importantly, as illustrated in the protein homology plots of Figure 11, we can discern no extended regions of identity or similarity between the *Synechococcus* lycopene cyclase and the bacterial ones. Yet these enzymes bind the same substrate and catalyze formation of the same product. Given the few sequences available, it is unclear to what degree the few limited regions of similarity suggested by the upper plot of Figure 11 reflect this commonality of enzymatic function or simply represent the random noise expected given the low stringency of the parameters chosen for the comparison.

Convergent Evolution of the Carotenoid Biosynthetic Pathways in Bacteria and Plants

The limited similarity between the two *Erwinia* lycopene cyclase enzymes and *Synechococcus crtL* gene product suggests that they have very different ancestries. Such lack of similarity is also the case for an earlier enzyme in the carotenoid biosynthetic pathway, phytoene desaturase. The *Synechococcus* phytoene desaturase is homologous to algal and plant gene products, that is, those in other oxygenic photosynthetic organisms (Bartley et al., 1991; Pecker et al., 1992), whereas phytoene desaturase gene products from fungi and photosynthetic bacteria (*crtI* genes) resemble those of other bacteria, such as the *Erwinia* species (Armstrong et al., 1993). We expect that lycopene cyclases from algae and plants will also prove to be homologous to the *Synechococcus* gene product, and preliminary experiments (J. Hirschberg, unpublished data) indicate that algae and plants contain genes that hybridize with a probe complementary to the *Synechococcus crtL* gene. Our

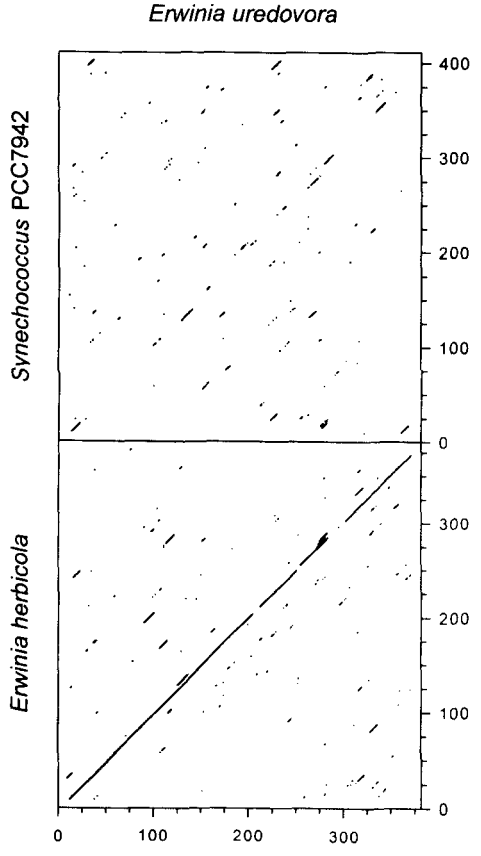

Figure 11. Protein Homology Plots of the Known Lycopene Cyclase Enzymes.

Amino acid sequences were compared over the entire length of the polypeptides for identities or similarities within a window of 15 residues. Nine identical or similar amino acids were scored as a dot. GenBank accession numbers for the *E. herbicola* and *E. uredovora* sequences are M87280 and D90087, respectively. The product predicted by a third *Erwinia crtY* gene sequence (GenBank M90698) differs very little from the *E. uredovora* gene product (data not shown).

results and observations provide additional support for our earlier hypothesis (Pecker et al., 1992, 1993) that the carotenogenic pathway of oxygenic plants, algae, and cyanobacteria arose independently of the pathway in bacteria, photosynthetic bacteria, and fungi. Together, these bacterial and plant-type pathways provide a classic example of convergent evolution.

METHODS

Organisms and Growth Conditions

Escherichia coli strain TOP10 (obtained from Invitrogen Corporation, San Diego, CA) was grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37°C in darkness on a platform shaker at 225 cycles per min. Media components were from Difco (yeast extract and tryptone) or Sigma (NaCl). Ampicillin at 150 μg/mL and/or chloramphenicol at 50 μg/mL (both from United States Biochemical Corporation) were used, as appropriate, for selection and maintenance of plasmids. The bleaching herbicide 2-(4-methylphenoxy)triethylamine hydrochloride (MPTA) was a gift of H. Yokoyama, Agricultural Research Service, United States Department of Agriculture, Pasadena, CA. MPTA was used at a final concentration of 40 μM and was added to the culture medium, immediately before inoculation, from a freshly made stock solution of 40 mM in methanol.

Plasmid Construction

Construction of plasmids pAC-LYC, pAC-NEUR, and pAC-ZETA is illustrated in Figure 12. The appropriate carotenoid biosynthetic genes from *Erwinia herbicola*, *Rhodobacter capsulatus*, and *Synechococcus* sp strain PCC7942 were cloned in the vector pACYC184 (obtained from New England BioLabs, Beverly, MA). An 8.6-kb BglII fragment containing the carotenoid biosynthetic genes of *E. herbicola* was obtained after partial digestion of plasmid pPL376 (Tuveson et al., 1986) and cloned in the BamHI site of pACYC184 to give the plasmid pAC-EHER (not shown). Deletion of adjacent 0.8- and 1.1-kb BamHI-BamHI fragments (deletion Z; Figure 12) and adjacent 1.6- and 0.6-kb AvaI-AvaI fragments (deletion XY) from pAC-EHER served to remove most or all of the coding regions of the genes for lycopene cyclase (*crtY*), β-carotene hydroxylase (*crtH*), and zeaxanthin glucosyltransferase (*crtX*). The resulting plasmid, pAC-LYC, retains functional genes for geranylgeranyl pyrophosphate synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*). Cells of *E. coli* containing this plasmid accumulated lycopene and formed pink colonies.

Plasmid pAC-PHYT (not shown) was constructed from pAC-EHER by deletion of adjacent 1.1-kb SalI-SalI and 2.0-kb SalI-BglII fragments (deletion XYI) as well as deletion Z. Cells containing this plasmid accumulated phytoene.

Plasmid pAC-ZETA was constructed by cloning a 2.2-kb PvuII-BamHI fragment containing the *Synechococcus* sp PCC7942 phytoene desaturase (*crtP*) gene in the blunted SalI site of pAC-PHYT. The *crtP* gene was actually a fusion with the N terminus of the *lacZ* gene and was excised from the plasmid pPDSdel35 (Linden et al., 1991). Expression of the gene is inducible with isopropyl-β-D-thiogalactopyranoside (IPTG), but sufficient enzymatic activity to support a visible accumulation of carotenoids in *E. coli* is obtained without the inducer. Cells of *E. coli* containing pAC-ZETA accumulated ζ-carotene and were pale yellow in color.

Plasmid pAC-NEUR was constructed by cloning a 3.7-kb EcoRV fragment from plasmid GABX2 (Armstrong et al., 1989), containing the phytoene desaturase gene from *R. capsulatus* (*crtI*), in the blunted SalI site of pAC-PHYT. Cells of *E. coli* containing pAC-NEUR accumulated neurosporene and formed yellow colonies.

Plasmid pTrcA-LCYKH (Figure 7) was constructed by cloning a 2.4-kb KpnI-HindIII fragment, containing the lycopene cyclase gene (*crtL*) of *Synechococcus* sp PCC7942 (open reading frame 3 [ORF 3] of Figure 3), in the KpnI and HindIII sites of the vector pTrcHisA (Invitrogen).

Complementation Mapping of the Lycopene Cyclase Gene

The complementation mapping of lycopene cyclase activity involved the cloning of various *Synechococcus* genomic fragments in the

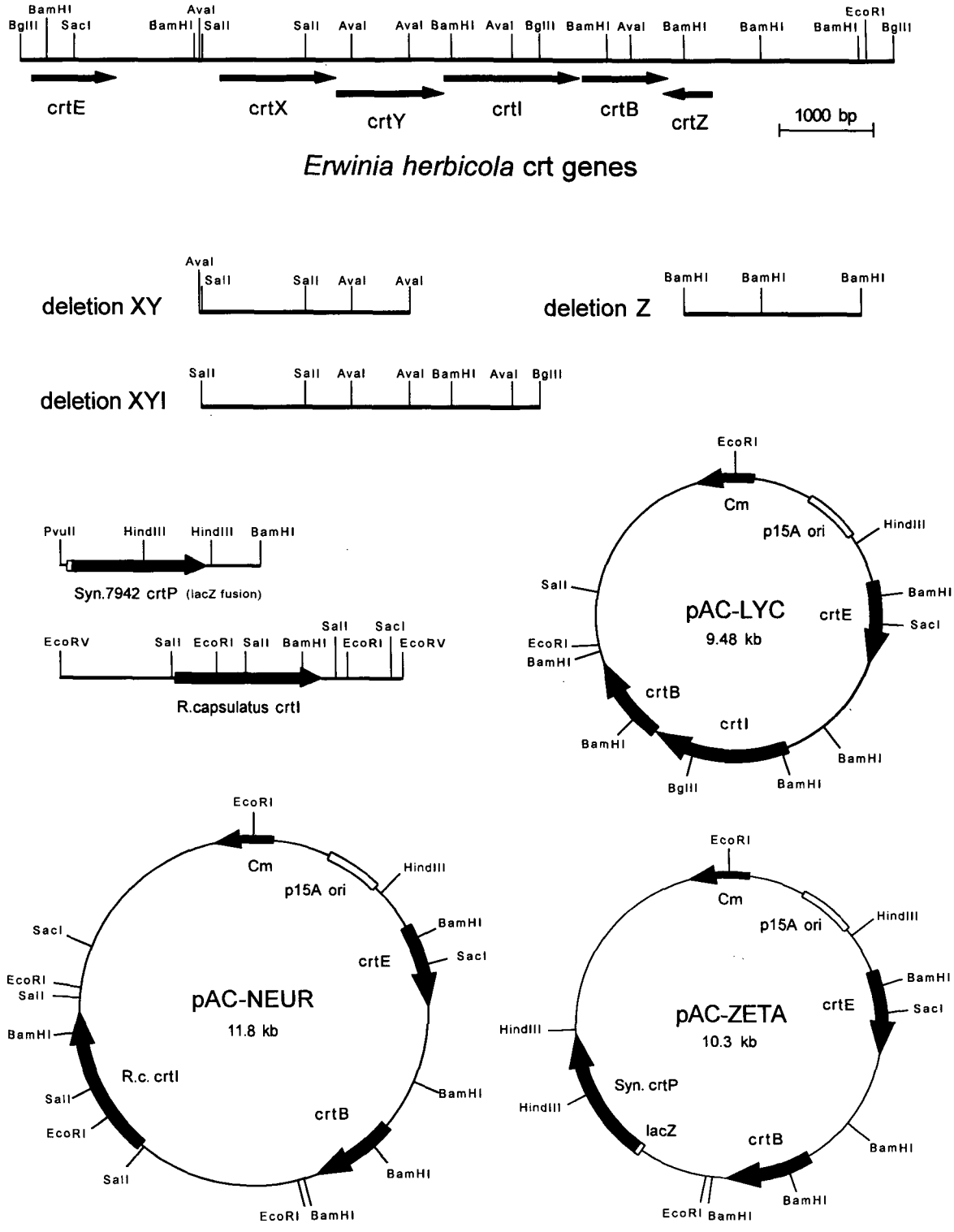

Figure 12. Construction of Plasmids pAC-LYC, pAC-NEUR, and pAC-ZETA.

A BglII-BglII fragment containing genes for the complete carotenoid biosynthetic pathway of *E. herbicola* was cloned in the BamHI site of the vector pACYC184. The three circular plasmids shown were created through a combination of the appropriate deletions of *E. herbicola* genes and insertion, in the blunted SalI site of the vector, of a fragment containing either the *Rhodobacter capsulatus* (R.c) *crtI* gene or the *Synechococcus* sp PCC7942 (Syn.) *crtP* gene (fused to the N terminus of the *lacZ* gene of pBluescript II KS+). Cm, chloramphenicol resistance gene from Tn*9*; p15A ori, origin of replication from plasmid p15A.

IPTG-inducible expression vector pTrcHisB (from Invitrogen) and introduction of the resulting plasmids in a lycopene-accumulating strain of *E. coli* (strain TOP10 containing the plasmid pAC-LYC). The closely related pTrcHisA and pTrcHisC were also used, in some cases, to clone in the other two frames. Aliquots of transformed cultures were plated on LB agar plates containing ampicillin (for selection of transformants with genomic subclones in the pTrcHisB vector) and chloramphenicol (for maintenance of the pAC-LYC plasmid). Petri plates were incubated at 37°C for 16 hr and then at room temperature for 2 to 3 days to allow maximum color development. Cultures containing only pAC-LYC formed pink colonies. A colony color of yellow or pinkish-yellow was considered positive evidence of lycopene cyclase activity. HPLC analysis (see below) was used to confirm the visual observations.

Sequence Analysis and Cloning Techniques

Nested deletions were created at both ends of a *Synechococcus* sp PCC7942 genomic EcoRI-BamHI fragment (Figure 3) cloned in the vectors pBluescript II KS+ and SK+ using the Erase-A-Base kit of Promega. Double-stranded DNA minipreps were made using the CTAB procedure of Del Sal et al. (1988) and sequenced using the Sequenase 2 kit of United States Biochemical Corporation. Both strands were sequenced completely, and both 2′-deoxyinosine-5′-triphosphate and 7-deaza-2′-deoxyguanosine-5′-triphosphate were used to resolve compressions. DNA from the *Synechococcus* mutant MPTA′-5 was sequenced from the XbaI site immediately upstream of ORF 2 to the end of ORF 3 using specific oligonucleotide primers synthesized for this purpose. Other procedures or methods used were performed according to manufacturer's protocols or standard methodologies (Sambrook et al., 1989) or have been described previously (Cunningham et al., 1993). DNA sequence analysis and searching of GenBank, Swiss-Prot, and PROSITE data bases were done using the Sequence Analysis Software Package, version 7.2-UNIX, of the University of Wisconsin Genetics Computer Group, Madison, WI. The Hitachi MacDNASIS program, version 3.0, was also used. The sequence reported in this paper has the GenBank accession number X74599.

Carotenoid Pigment Analysis by HPLC

A 0.5-mL aliquot of an overnight culture was used to inoculate 50 mL of LB in a 250-mL Erlenmeyer flask. Cultures were grown (see details above) for 24 hr in darkness and then harvested by low-speed centrifugation in 15-mL disposable conical centrifuge tubes. The pellets were resuspended with ~0.5 mL of water, and 10 mL of 6% KOH in methanol was then added. The air over the solution was replaced with nitrogen gas, and the tubes were capped tightly and stored in darkness at room temperature for 4 hr. After this saponification step, insolubles were pelleted by centrifugation and the pellets were extracted with methanol, then with methanol/diethyl ether, 1:1 (v/v), and finally with diethyl ether until the pellets were colorless. The extracts were combined, and the carotenoid pigments were transferred to diethyl ether in a separatory funnel and washed free of alkali according to standard procedures (Jensen and Jensen, 1971). The diethyl ether extracts were evaporated to dryness under a stream of nitrogen and resolubilized in the HPLC mobile phase. Samples were injected onto a 4.6 mm × 15 cm Ultrasphere ODS column (5 μM particle size; from Beckman Instruments, Inc., Berkeley, CA) with a mobile phase of acetone/water, 100:4 (v/v), a flow rate of 0.8 mL per min, and a 20-μL sample loop. The detector was set to a wavelength of 450 or 425 nm, as required, and the flow cell was simultaneously monitored at 375 nm. Spectra of eluted pigments were recorded in hexane after evaporation of the mobile phase under a stream of nitrogen gas. Spectra were recorded at a scan rate of 120 nm/min with a bandwidth of 1 nm and data points at 0.5 nm intervals. All procedures were performed under dim room lights as much as possible. Lycopene purified from tomato fruit and synthetic β-carotene (Sigma) were used as reference standards. Spectra of unknown peaks were compared with those of authentic compounds and with spectral properties of known compounds in hexane (listed by Davies, 1976).

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